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Optimization of Sulforaphane Separation from Broccoli Seeds by Macroporous Resins

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Abstract: In this study, the adsorption and desorption properties of sulforaphane on macroporous resins (HP20, SP207, SP850, and HP2MGL) were investigated. Analysis revealed that SP850 resin was most effective in the separation of sulforaphane. The equilibrium experimental data obtained at different temperatures were well fitted to the Langmuir and Freundlich isotherms. To optimize the separation process, dynamic adsorption and desorption tests were performed with a column packed with SP850 resin. The results showed that the optimum parameters for adsorption were as follows: flow rate: 5 BV/h, pH 2, temperature: 25°C; for desorption: ethanol–water (40:60, v/v), 6 BV as an eluent, flow rate: 6 BV/h. The highest purity of sulforaphane product was 85.9%, i.e., 107-fold higher than those in broccoli seeds through one run treatment on the column packed with SP850 resin under normal conditions. This indicated the high efficiency of SP850 resin in separating sulforaphane.

Keywords: Optimization, sulforaphane, broccoli, macroporous resins

INTRODUCTION

Cruciferous vegetables contain compounds that have the potential to fight cancer. Numerous studies have shown that the anticarcinogenic effect of cruciferous vegetables is related to their unique content in a large variety of

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glucosinolates (1). When vegetables are grounded or chopped, myrosinase enzyme (thioglucoside glucohydrolase, EC3.2.3.1) and glucosinolates come into contact. Myrosinase breaks the β-thioglucoside bond of glucosinolate molecules, producing glucose, sulfate, and a diversity of aglycone products. The resultant aglycones then undergo non-enzymetic, intramolecular rearrangement to yield isothiocyanates, thiocyanates, or nitriles. Sulforaphane (4-methylsulfinybutyl isothiocyanate), an isothiocyanate derived from glucoraphanin, was initially identified as the principal inducer of Phase II enzymes (2) and subsequently shown to possess anticarcinogenic activities (3–5). Epidemiologic studies have demonstrated the inverse associations between crucifer intake and the incidence of brain (6), lung (7), pancreas (8), bladder (9), prostate (10), ovarian (11), skin (12–14), stomach (15), and colon cancer (16). Sulforaphane has been of increasing interest for nutraceutical and pharmaceutical industries due to its anticarcinogenic effect. As a conclusion, the above-mentioned properties make sulforaphane an attractive multipotent anti-tumor agent, providing an important new approach for future research on clinical applicability of sulforaphane to cancer patients.

Sulforaphane is a promising molecule for fighting cancer. However, an optimum purification technique for the collecting of sulforaphane has little reported yet. Pure sulforaphane can be prepared by chemical synthesis (17, 18) or purification from plant (19–21). However, several highly toxic substances were involved in chemical synthesis, and further purification was required to remove such substance. Moreover, purification of sulforaphane from plants are usually performed with preparative reverse phase high performance liquid chromatography (RP-HPLC) which is very expensive and requires elaborate pre-separation of the sample to remove a large quantity of contaminants. So it is improper for the purification of sulforaphane in the industry process. In addition, low-pressure column chromatography (LPCC) for purification with the silica gel as packing material can be effective, but raw materials must be extracted from broccoli seeds meal with immiscible solvent, leading to a loss of the derived compound. Moreover, the loading capacity also is lower than 0.5 g/60 g silica gel. Thus, it is important to employ new methods to solve this problem. Macroporous resins are durable polar, non-polar, or slightly hydrophilic polymers have high adsorption capacity with possible recovery of the adsorbed molecules, relative low cost, and easy regeneration. With the progress of synthesis technology of macroporous resins, the adsorption method with macroporous resins has been widely used in the field of natural substances extraction such as drugs (22) and polyphenols (23–29). However, up to date, studies on adsorption of sulforaphane onto macroporous resins have not been reported. Herein, macroporous resins were used to separate the sulforaphane.

In this paper, the adsorption and desorption properties of sulforaphane on different macroporous resins have been investigated. A novel method was proposed to purify sulforaphane with optimal macroporous resin and solvents that are easy to be recovered. Certain parameters such as pH value

of sample, flow rate, temperature, elution modes, etc. were also optimized to ensure the separation efficiency.

EXPERIMENTAL

Materials

Broccoli seeds were kindly provided by Vegetables and Flowers Institute of China Academy of Agriculture Science. Sulforaphane standard was purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile was HPLC grade. Ethanol, ethyl acetate, anhydrous calcium carbonate were of analytical grade purchased from Beijing chemistry company (Beijing, China). Deionized water was purified by a Milli-Q Water Purification system (Millipore, MA, U.S.A.). The Coomassie brilliant blue G-250 (CBB G-250, Shanghai Chemical Reagent Company) solution for spectrophotometric determination of protein was prepared according to the common procedure. All solutions prepared for HPLC were subject to filtration through 0.45 μm nylon membranes. Macroporous resins including HP20, SP207, SP850, and HP2MGL were purchased from Mitsubishi Chemical Corporation (Tokyo, Japanese). The physical properties of macroporous reins are summarized in Table 1.

Preparation of Broccoli Seeds Extracts

Fifty grams of seeds was homogenized in an analytical grinder. Ground seed meal was added to 200 ml of pure water, and the mixture was allowed to autolyze for 2 h at 25°C. This paste was centrifuged at 18,000 rpm for 15 min by a centrifuge (Beckman Coulter. Inc. U.S.A.). The residue was dissolved in 100 ml deionized water and centrifugated again. The extracted solutions were mixed and quantitatively analyzed by HPLC.

Table 1. Physical properties of the test macroporous resins

Type	HP20	SP850	SP207	HP2MG
Chemical structure	Aromatic	Aromatic	Modified aromatic	Methacrylic
Apparent density (g/l-R)	680	670	780	720
Water content (%)	55–65	46–52	43–53	55–65
Pore volume (ml/g)	1.3	1.2	1.3	1.2
Surface area (m^2/g)	600	1000	6000	500
Pore radius (A)	>200	38	110	200

Removal of Protein

Acid was adjusted to various pH values. In order to remove protein, these samples at different pH were centrifuged at 18,000 rpm for 15 min by a centrifuge (Beckman Coulter. Inc. U.S.A.). The traditional Coomassie brilliant blue G-250 spectrophotometric method (30) has been used for the quantitative determination of proteins.

Analysis of Sulforaphane by HPLC

Sulforaphane was analyzed by a Hitachi HPLC apparatus equipped with Hitachi model L-7100 pumps, L-7420 tunable absorbance detector, reversed-phase C_{18} column (250 × 4.6 mm, 5 μm, Diamodsil^{TM}). The solvent system consisted of 20% acetonitrile in water, and changed linearly over 10 min to 60% acetonitrile, and maintained 100% acetonitrile for 2 min to purge the column. Column oven temperature was set at 30°C. The flow rate was 1 ml/min, and 10 μl sample was injected into the column. Sulforaphane was detected by UV 254 nm.

Static Adsorption and Desorption Tests

The static adsorption tests of broccoli seeds extracts were carried out as follows:

1 ml hydrated test resin and 50 ml sample solution of broccoli seeds extracts was added into a flask with a lid. The mixtures were continuously stirred at 100 rpm for 24 h and kept at room temperature 30°C by thermostatic bath. The adsorption solution was analyzed by HPLC. The desorption process was conducted as follows: the resin was first washed with deionized water after reaching adsorption equilibrium and then desorbed with 50 ml ethanol solution. The flask was shaken (100 rpm) for 24 h at a constant temperature of 30°C. The desorption solution was analyzed by HPLC. The selectivity of the resin was based on its adsorption capacity and the ratio of adsorption and desorption. The adsorption and desorption property were determined at various pH.

The tests for adsorption isotherms of sulforaphane on the selected SP850 resin at various temperatures were also conducted by mixing 50 ml sample solutions of broccoli seeds extracts at different concentrations with preselected resins in shakers at temperature 25°C, 30°C, and 35°C. These experimental data were fitted to the Freundlich and Langmuir equations by calculations.

Dynamic Adsorption and Desorption Tests

Dynamic adsorption and desorption experiments were performed in the glass columns (10 mm × 200 mm) wet-packed with SP850 resin. The bed volume (BV) of resin was 10 ml, and the packed length of resin bed was 13 cm.

Sample solution flowed through the glass column at a certain flow rate and the sulforaphane content in the effluent liquid were monitored by HPLC analysis.

Upon reaching adsorptive equilibration, the adsorbate-laden column was first washed with deionized water and then eluted by ethanol–water (20:80, 60:40 v/v) solution at a certain flow rate. The test for the content of sulforaphane in desorption solution was carried out by HPLC. The ethanol–water (20:80 v/v) solution was 3 BV and the ethanol–water (60:40 v/v) solution was 6 BV. The volume of the sample solution was maintained at 500 ml with a concentration of sulforaphane 0.7 mg/ml. The effect of the sample flow rate on the adsorption capability and desorption flow rate on the desorption capability were studied.

Elution Modes Tests

The best sample flow rate was determined as 5 BV/h, and the elution flow rate was 1 ml/min. The gradient elution modes tests were carried out as follows:

When adsorption reach the equilibration, three kinds of elution modes were carried out with deionized water followed by the ethanol–water (20:80, v/v) and A solvent systems. The ethanol–water (20:80, v/v) was 3 BV to wash the impurities of low polarity. The eluent A include ethanol–water (40:60,v/v) in Mode 1, ethanol–water (50:50,v/v) in Mode 2 and ethanol–water (60:40, v/v) in Mode 3, respectively. The volume of each eluent A was 6 BV. Finally, ethanol was used to wash the adsorbate-laden column. Each portion of the desorption solutions was analyzed by HPLC.

The sulforaphane eluent was removed from ethanol solvent in a rotary evaporator (RE-52AA, Shanghai Huxi Instrument Co., China) at 30°C and extracted twice with 30 ml ethyl acetate. The ethyl acetate fraction was dried at 30°C under vacuum on a rotary evaporator. Purity and recovery ratio of the sulforaphane product were determined with HPLC.

Calculation of Adsorption Capacity, Ratios of Adsorption, and Desorption

The following equations were used to quantify the adsorption capacity as well as the ratios of adsorption and desorption. Adsorption evaluation:

$$q_e = \frac{(C_0 - C_e) \times V}{V_S} \tag{1}$$

$$E = \left(\frac{C_0 - C_e}{C_0}\right) \times 100\% \tag{2}$$

Where the q_e (mg/ml) is the adsorption capacity at adsorption equilibrium point, C_0 and C_e are the initial and equilibrium concentrations of sulforaphane

in solutions, respectively (mg/ml), V is the volume of solutions, V_S is the volume of resins, E is the adsorption ratio (%) which is the percent of the quantity adsorbed to the initial quantity under equilibrium. Desorption evaluation:

$$D = \left(\frac{C_d \times V_d}{(C_0 - C_e) \times V} \right) \times 100\% \quad (3)$$

Where D is the desorption ratio (%), C_d is the concentration of sulforaphane in the desorption solution (mg/ml), V_d is the volume of the desorption solution (ml), C_0, C_e and V are the same as described above.

RESULTS AND DISCUSSION

Adsorption and Desorption Capacities, Desorption Ratio

As shown in Table 2, the adsorption capacity and the desorption capacity of SP207 and SP850 resins towards sulforaphane were considerably higher than those of other resins, which was attributed to the capabilities of the resins and the chemical features of the adsorbed substance. Pore size is a key parameter describing the adsorptive charaterictics of synthetic adsorbents. Comparison of the pore size of macroporous resins in Table 1 indicated that SP207 and SP850 have the smaller pore, demonstrating that the smaller pore can adsorb sulforaphane more effectively. SP850 resin has the smallest pore radius, so SP850 resin was selected to further investigate their adsorption behavior towards sulforaphane.

Effect of Sample Solution pH

The pH influences the extent of the solutes ionization, thus affecting the affinity of the solutes and solutions. As shown in Table 3, the adsorption

Table 2. Results of adsorption capacities (mg/ml resin), adsorption and desorption (%) of different resins

Name	Adsorption capacity (mg/ml resin)	Adsorption (%)	Desorption (%)
HP20	17.2	53.7	83.3
SP207	28.5	81.5	90.9
SP850	29.7	82.5	93.4
HP2MGL	6.1	23.0	68.9

Condition: The hydrated test resin was 1 ml. The sample solution of broccoli seeds extracts was 50 ml. The temperature was set at 30°C.

Table 3. Effects of different pH of the sample solution

pH	Sulforaphane (mg/ml)	Proteins (mg/ml)	Adsorption capacity (mg/ml resin)	Adsorption (%)
2	0.701	0.081	28.9	80.3
3	0.701	0.129	27.5	76.5
4	0.699	0.604	29.8	82.8
5	0.697	3.594	29.7	82.7

Condition: The SP850 resin was 1 ml. The sample solution of broccoli seeds extracts was 50 ml. The temperature was set at 30°C.

capacity of sulforaphane on SP850 resin at different pH values were nearly the same. The pH of the extract had no effect on the efficiency of adsorption, which confirmed that sulforaphane was stable in acid. Sample solutions contain large amounts of proteins and other impurities. As pH increased, the concentration of proteins enhanced. This indicated that low pH was advantageous to the present process. Thus, pH 2 was selected for the following tests.

Adsorption Isotherms

Equilibrium adsorption isotherms were constructed at the temperature of 25°C, 30°C, and 35°C. The initial concentrations of sulforaphane were 0.0867, 0.1733, 0.2600, 0.3467, 0.4333, and 0.5200 mg/ml, respectively. The effect of the temperature within the range from 25°C to 35°C is showed in Fig. 1. It can be seen that a rise in temperature reduced the

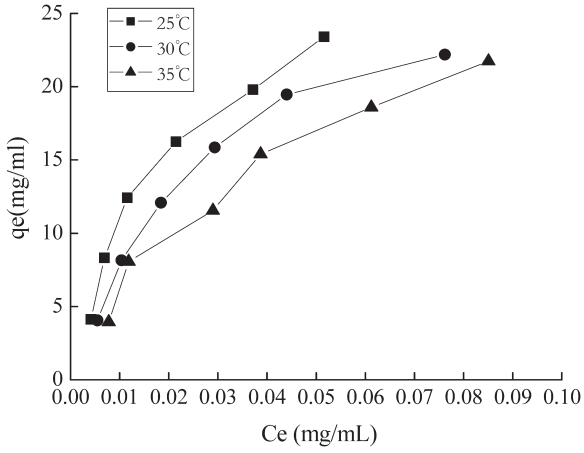

Figure 1. Adsorption isotherms for sulforaphane on SP850 resin at 25°C, 30°C, and 35°C.

adsorption capacity of the adsorbents and reduced the time to reach equilibrium. The results indicated that the adsorption process was exothermic and low temperature condition was advantageous to present process. As the temperature increased, the adsorption rate decreased as a result of the restriction by the occurrence of adsorption at high temperature and thus a rapid equilibrium.

The Langmuir equation is an isotherm equation most widely used for modeling the adsorption equilibrium data. The Langmuir equation is based on a theoretical model where the maximum adsorption capacity corresponds to a monolayer saturated with adsorbate molecules on the adsorbent surface, which is energetically homogeneous.

$$q_e = \frac{aC_e}{1 + bC_e}, \quad a = q_{\max} k_l, \quad b = k_l \tag{4}$$

Where q_{max} is a constant related to the adsorptive capacity, k_l is the parameter which relates to the adsorption energy.

$$k_l = k_l^{\infty} \exp\left(-\frac{\Delta H_l}{RT}\right) \tag{5}$$

A linearized form of Eq. (4) can be written as:

$$\frac{C_e}{q_e} = \frac{1}{k_l q_{\max}} + \frac{C_e}{q_{\max}} \tag{6}$$

The Langmuir equation was converted to the linearized form with C_e and C_e/q_e as independent variable, the experimental data were statistically analyzed and R^2 was obtained.

The Freundlich equation is an experiential model which is widely used in fluid-solid adsorption system. The experimental data were fitted to the Freundlich Eq. (7), describing the interaction of solutes with the resins:

$$q_e = k_f C_e^{\frac{1}{n}} \tag{7}$$

Where k_f and n are both the Freundlich constants. k_f reflects the adsorption capacity of the adsorbent and n reflects the adsorption affinity of the adsorbent to the adsorbate.

A linearized form of Eq. (7) can be written as:

$$\ln q_e = \ln k_f + 1/n \ln C_e \tag{8}$$

The k_f and n values can be obtained from the intercept and slope, respectively, and the linear regression line from a plot of ln q_e versus ln C_e. The Langmuir and Freundlich parameters were summarized in Table 4.

It can be seen that the correlation coefficients (R^2) of both the Langmuir and the Freundlich equations on SP850 resin were >0.9300. The correlation coefficient of Langmuir equation at 30°C was the highest of 0.9830. In the Freundlich equation, the values of n in methanol were all higher than 1, indicating that the adsorption in methanol was favorable. The values of k_f were very high,

Table 4. Langmuir and Freundlich adsorption parameters of sulforaphane on SP850 resin at different temperatures

Temperature (°C)	Langmuir equation	R^2	Freundlich equation	R^2
25	$C_e/q_e = 0.02933C_e + 0.00071$	0.9636	$q_e = 170.0C_e^{0.6326}$	0.9304
30	$C_e/q_e = 0.03072C_e + 0.00101$	0.9830	$q_e = 138.3C_e^{0.6416}$	0.9482
35	$C_e/q_e = 0.02868C_e + 0.00149$	0.9367	$q_e = 119.7C_e^{0.6591}$	0.9432

demonstrating the adsorption of sulforaphane on SP850 resin and its advantage to separate sulforaphane. It is clear that a rise in temperature could reduce the value of k_f, which further confirmed that high temperature restricted the occurrence of adsorption and the present adsorption was exothermic.

Effect of Sample Flow Rate on the Capacity of Adsorption

Macroporous resins have a large surface area and fine pore structures inside the particle like activated carbon. For this porous characteristic, they can effectively adsorb organic compounds from aqueous solutions. When the adsorption reaches the breakthrough point, the adsorption effect decreases and even disappears. So it is important to set up the breakthrough point in order to calculate the quantity of resin, the processing volume of sample solution and the proper sample flow rate. The results were shown in Fig. 2, and Table 5.

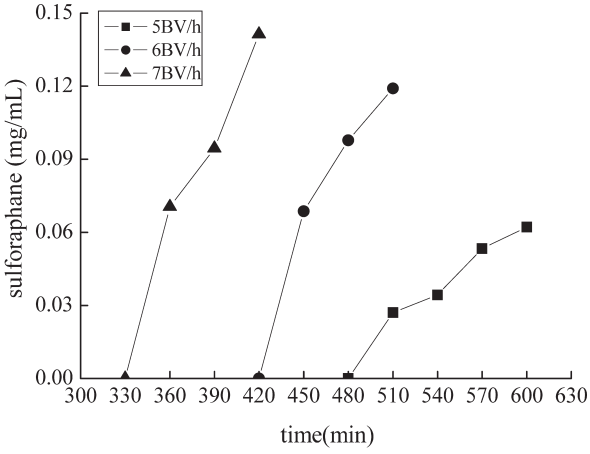

Figure 2. Effect of sample flow rate on the capacity of adsorption. Condition: The column was a 10 mm × 200 mm glass column wet-packed with 10 ml SP850 resin. The volume of sample solution was maintained at 500 ml with a concentration of sulforaphane 0.7 mg/ml.

Table 5. Effects of different flow rates on the adsorption capacities of sulforaphane

Flow rate (BV/h)	Adsorption capacity (mg)	Adsorption (%)	Breakthrough point (min)
5	345.20	98.6	480
6	335.25	95.8	420
7	320.72	91.6	330

Condition: The column was a 10 mm × 200 mm glass column wet-packed with 10 ml SP850 resin. The volume of sample solution was maintained at 500 ml with a concentration of sulforaphane 0.7 mg/ml.

As shown in Table 5, the best adsorption performance was obtained at the lowest flow rate 5 BV/h, which is likely due to better particle diffusion in sample solutions. Smaller solutes can penetrate into the particle by diffusing through the pores, when a solution is allowed to contact with adsorbent particles. The lowest flow rate 5 BV/h allowed this to happen. An even lower flow rate prolonged the working period. Therefore, 5 BV/h was selected as the best sample flow rate for further experiments.

Dynamic Desorption Curve on SP850 Resin

The dynamic desorption curves using SP850 resin were obtained based on the volume of desorption solution and the sulforaphane concentration in the desorption solution (Fig. 3). It is important to choose a proper flow rate to desorb

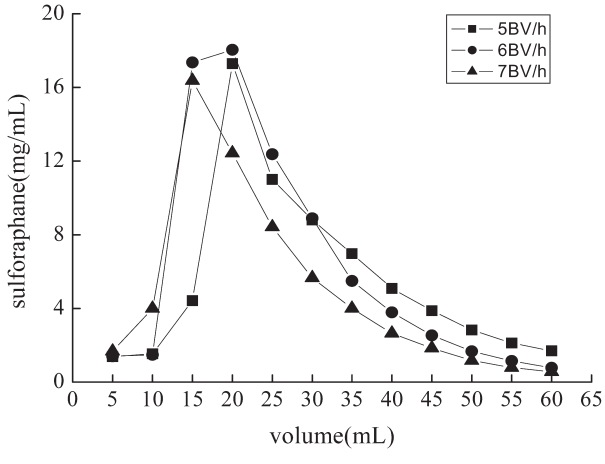

Figure 3. Dynamic desorption curves of sulforaphane on column packed with SP850 resin at different desorption flow rates. Condition: The column was a 10 mm × 200 mm glass column wet-packed with 10 ml SP850 resin. The elution were carried out with deionized water followed by the ethanol–water (20:80, v/v) solution and the ethanol–water (60:40, v/v) solution.

Table 6. Effects of different flow rates on the desorption capacities of sulforaphane on SP850 resin

Flow rate (BV/h)	Desorption capacity (mg)	Purity (%)
5	341.80	76.2%
6	337.43	76.4%
7	303.10	76.4%

Condition: The column was a 10 mm × 200 mm glass column wet-packed with 10 ml SP850 resin. The elution were carried out with deionized water followed by the ethanol–water (20:80, v/v) solution and the ethanol–water (60:40, v/v) solution.

sulforaphane from resin effectively. As can be seen in Table 6, the purity of sulforaphane is nearly equal. In the dynamic desorption test, the desorption performance at 5 BV/h was the best. However, at this desorption flow rate, the working time was too long. Therefore, 6 BV/h was chosen as the optimal desorption flow rate in consideration of the short working time and the highest desorption ratio. Approximately 6 BV of desorption solution could completely desorbed sulforaphane from SP850 resin when flow rate was 6 BV/h.

Effect of Elution Modes on the Recovery Ratio and the Purity of Sulforaphane

In order to reduce the consumption of reagents and improve the desorption efficiency, various elution modes were carried out under the following conditions: the concentration and volume of sulforaphane in sample solution was 0.7 mg/ml and 50 BV respectively during adsorption process. Desorption process was conducted as described in Section 2.7. The results were shown in Table 7.

As the ethanol in water solutions increased, the desorption capacity of the eluent enhanced accordingly and reached a peak at the concentration of 60%.

Table 7. Effects of elution modes on the recoverey and purity of sulforaphane

Elution modes	Desorption (%)	Purity (%)	Recovery (%)
Mode 1	91.3	85.9	80.3
Mode 2	94.6	74.9	82.9
Mode 3	96.1	76.4	84.4

Condition: The column was a 10 mm × 200 mm glass column wet-packed with 10 ml SP850 resin. The flow rate was 6 BV/h. The elution modes were carried out with deionized water followed by the ethanol–water (20:80, v/v) and A solvent system. The eluent A was ethanol–water (40:60, v/v) in mode 1, ethanol–water (50:50, v/v) in mode 2 and ethanol–water (60:40, v/v) in mode 3, respectively.

After removal of the ethanol solvent, the extraction was conducted twice with 30 ml ethyl acetate which was then dried for the purification and the recovery of sulforaphane product. As shown in Table 7, the purity of sulforaphane was more than 70% under conditions of the three elution modes, and it was 85.9% in elution mode 1 which was the highest as compared to those in the others.

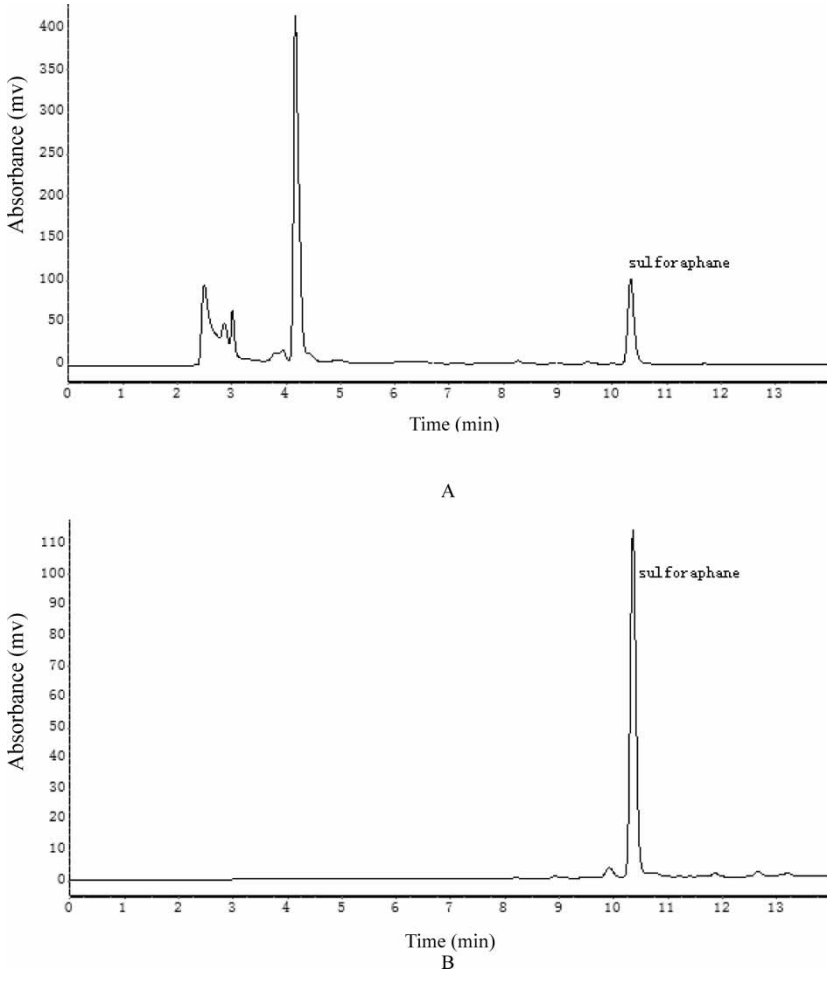

Figure 4. Chromatograms of sample solution before (A) and after (B) separation on a column packed with SP850 resin. Condition: Sulforaphane was detected by UV 254 nm. The colume was reversed-phase C_{18} column (250 × 4.6 mm, 5 μm, Diamodsil^{TM}). The solvent system consisted of 20% acetonitrile in water, and changed linearly over 10 min to 60% acetonitrile, and maintained 100% acetonitrile for 2 min to purge the column. Column oven temperature was set at 30°C. The flow rate was 1 ml/min, and 10 μl sample was injected into the column.

It can probably be explained as a result of different polarity of these three solvents. The ethanol–water (40:60 v/v) solution has the similar polarity with sulforaphane, so sulforaphane was desorbed much more than the impurity. The chromatograms of the test samples before and after treatment with SP850 resin were shown in Fig. 4. By comparison, it can be seen that some impurities were removed and the relative peak area of sulforaphane increased obviously after the separation on SP850 resin. Higher purity and recovery of the sulforaphane products could be obtained if the smaller diameter and higher efficient resin or more complex solvent system was used.

CONCLUSIONS

The separation of sulforaphane with macroporous resins was successfully achieved in this study. Among the four investigated resins, SP850 resin offers the best separation performance of sulforaphane. Its adsorption experimental data were fitted better to the Langmuir model than the Freundlich model. Crude sulforaphane extracted from broccoli seeds meal after removal of proteins was used as raw materials to prepare highly purified sulforaphane on macroporous resin. The samples do not require much treatment before purification. The best sample flow rate was 5 BV/h and its adsorption ratio was 98.6%. After reaching adsorptive equilibration, deionized water and ethanol–water (20:80, v/v) solution were employed for removal of impurities followed by elution with ethanol–water (40:60, v/v) solution at 6 BV/h. The sulforaphane eluent was removed from the ethanol solvent in a rotary evaporator and extracted twice with 30 ml ethyl acetate. After the ethyl acetate was dried, the purity of the sulforaphane product was 85.9%, which is 107-fold higher than those in broccoli seeds. Compared to conventional separation method of sulforaphane, this adsorption method is convenient because of its procedural simplicity, lower cost, and high efficiency, and it can be referenced for large-scale separation of sulforaphane from crude seed extracts.

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